IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re PATENT APPLICATION of:

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Appln. No. 08/966,223 Group Art Unit: 1645

Filed: November 7, 1997 Examiner: M.P. Allen

FOR: GDF-1

* * *

RULE 132 DECLARATION

I, Ted Ebendal, declare and state as follows:

(1) I reside at Börjegatan 45 B, S-752 29 Uppsala, Sweden.

(2) I am a Professor and Chairman in the Department of Developmental Biology, Faculty of Medicine, Uppsala University since 1988. I hold a Ph.D. (Doctor of Philosophy) degree which was earned from Uppsala University, Sweden in 1976. A copy of my *curriculum vitae* is attached.

(3) I am an author of over 100 peer-reviewed publications in the field of neuronal growth factors and neurotrophic factors.

(4) Recombinant human GDF-1 (rhGDF-1) was provided by Michael Jarpe of Cambridge NeuroScience for use in the assays reported herein.

(5) On information and belief, rhGDF-1 was produced as follows. The cDNA of human GDF-1 (amino acid residues 255 to 373) was cloned into pRSET (Invitrogen). The construct was designed to produce a fusion protein which adds 34 amino acid residues to the N-terminus of rhDGF-1 including six histidine residues. There is an enterokinase cleavage site between the N-terminal extension and the rhGDF-1 sequence to facilitate removal of the tag. However, this extension was not removed for the assays reported herein.

(6) On information and belief, the above-described expression construct was inserted into the *E. coli* cell line BL21(DE3)pLysS to induce rhGDF-1 expression. Expression was induced by the addition of IPTG and was allowed to proceed for 4 hours. rhGDF-1 was produced in inclusion bodies.

(7) On information and belief, the inclusion bodies containing rhGDF-1 were solubilized and folded in 6 M guanidine and 100 mM dithiothreitol. Reducing agent and denaturing agent was removed by reverse phase HPLC. The protein was dried down in a Speed Vac and resuspended in 8 M urea at 5 mg/ml protein concentration. The protein solution was then diluted 1/100 to a final concentration of 50 µg/ml in refolding buffer of 10 mM reduced glutathione, 1 mM oxidized glutathione, and 50 mM Tris buffer (pH 9.0). The rhGDF-1 protein was allowed to refold for 20 hours at 25°C.

(8) On information and belief, a sample of the refolded rhGDF-1 protein was then analyzed by reducing and non-reducing SDS-PAGE. The gel was stained with Coomassie and the proportion of dimer was determined by densitometry. The rhGDF-1 dimer was found to be approximately 20% of total protein. The rhGDF-1 preparation was stored at -80°C.

(9) The following assays were performed under my direction and the results were analyzed by me.

(10) The sample was assayed in a fibre outgrowth assay using sympathetic ganglia from embryonic day 9 chicken embryo explanted into a collagen gel. See Ebendal *et al.*, Journal of Neuroscience Research, vol. 40, pp. 276-284 for a description of the use of explanted ganglia in collagen gels. Neurotrophin-3 (NT-3) only weakly stimulates sympathetic fibre outgrowth in this assay (see panel d of Fig. 4 in Ernfors *et al.*, Proceedings of the National Academy of Science, U.S.A., vol. 87, pp. 5454-5458). Members of the TGF-beta superfamily of proteins potentiate the effects of NT-3 in this assay.

(11) The sample of GDF-1 was diluted 100-fold and then further diluted in culture medium with 1% fetal calf serum as a carrier. GDF-1 was assayed on sympathetic ganglia at a

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concentration of 2.5 to 250 ng/ml. The ganglia were examined after two days of incubation using darkfield microscopy. No fibre outgrowth was evoked by GDF-1 at any of these concentrations.

(12) Therefore, the potentiating effect of GDF-1 on neurotrophin activity could be assessed by comparing fibre outgrowth induced by NT-3 in the presence or the absence of GDF-1. Any increased fibre outgrowth caused by the combination of NT-3 and GDF-1 would be due to potentiation, instead of the effects of GDF-1 alone.

(13) The potentiating effect of GDF-1 in the sympathetic fibre outgrowth assay (Ernfors *et al.*, *id.*) was determined with human NT-3 (Austral Biologicals) at a concentration of 2 ng/ml and GDF-1 at concentrations between 0 to 250 ng/ml. Fibre outgrowth density was scored in a blinded fashion by two independent observers with culture dishes arranged in random order. Scores were recorded on a scale from 0 (no fibres) to 5 (very high density of fibres forming a circular halo around the explanted nervous tissue). The assay was repeated three times. The results below represent the mean of the scores given for each culture by each observer.

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	GDF-1 concentration	Mean Score
Medium Only	0 ng/ml	0.0
NT-3 alone	0 ng/ml	1.7
GDF-1 alone	250 ng/ml	0.0
NT-3 + GDF-1	250 ng/ml	3.1
NT-3 + GDF-1	50 ng/ml	2.3
NT-3 + GDF-1	5 ng/ml	1.8

(14) The combination of GDF-1 at 250 ng/ml with NT-3 shows a significant potentiation effect in comparison to the response obtained with NT-3 alone (statistically significant difference at $P < 0.001$ using Mann-Whitney U test). There is also a clear trend of potentiation of NT-3 by GDF-1 at 50 ng/ml, although this difference is not statistically significant in the present format of the assay.

(15) The specific activity of GDF-1 in the assay shows a reasonable dose-response relationship between 50 to 250 ng/ml.

(16) In view of the above results, I conclude that GDF-1 has biological activity on neurons similar to members of the TGF-beta superfamily of proteins.

(17) I declare further that all statements made herein of my own knowledge are true and that all statements made on

information and belief are believed true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

x Ted Ebendal
Ted Ebendal

x April 9, 1998
Date

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CURRICULUM VITAE for TED EBENDAL

Born: September 21, 1948. Stockholm, Sweden
Sex: Male
Marital status: Married, 1 child (born 1974)
Address: Börjegatan 45 B, S-752 29 UPPSALA, Sweden

Education/academic degrees:

1971 Bachelor of Science. Uppsala University, Sweden
1972 Master of Science, Uppsala University
1976 Doctor of Philosophy, Uppsala University
1977 Docent in Zoology, Uppsala University
1987 Professor, Faculty of Medicine, Uppsala University

Professional Experience:

1972-76	Teaching Instructor in Zoology, Uppsala University
1977	Assistant Professor in Zoology, Uppsala University
1977	Visiting scientist at Strangeways Research Laboratory, Cambridge, England
1977-81	Docent (Associate Professor) appointment in Zoology, Uppsala University
1981-87	Research Associate Professor in Neurobiology at the Swedish Natural Science Research Council
1988-	Full Professor of Developmental Biology, Faculty of Medicine, Uppsala University

Scientific Awards: King Oscar Prize, Uppsala University (1982). Erik K. Fernström's prize to especially promising young scientists (1991).

Invited Oral Presentations have been given at about 80 international meetings.

Current research interests: Molecular and developmental neuroscience, development and repair mechanisms in the nervous system, neuronal growth factors and their receptors including NGF, NT-3, GDNF and BMPs. Homologous recombination in transgenic mice using embryonic stem cells.

Administrative and scientific duties at the Faculty of Medicine, Uppsala University
Chairman at the Dept of Developmental Biology (1988-). Member of the Medical Faculty Board (1988-93). Member of various scientific priority committees etc (1988-), Vice Chairman of Neuroscience Center at Uppsala University, (1989-1995). Chairman Uppsala University Animal Research Board (1997-)
Pre-doctoral advisor: Supervised 11 PhD students of which 8 have finished their PhD thesis and three are on the way.
Post-doctoral advisor for: Wilma Friedman (USA, 1986-88), Reg Williams (Australia, 1991-94).

Organization of scientific meetings, courses etc: Organized EMBO and BMC Summer School courses for graduate students in Uppsala and at the Karolinska Institute and participated in the organization committees for international meetings in Sweden and abroad (ISDN Biennial Meeting in Tampere 1996, 5th NGF Meeting 1998).
Referee assignments etc: Exp. Cell Res., Exp. Brain Res.
Associate editor: J. Neurosci. Res., Int. J. Dev. Neurosci, Neuron, Alzheinmer's Disease.
Reviewer of applications for grants and research positions: Uppsala University, Karolinska Institute, Stockholm University, Umeå University. MRC Sweden, NSF USA and various Swedish Universities.
Member of scientific committees: The Swedish Foundation for Brain Research (1995-), The International Human Frontier Science Program (Brain Functions 1996-), The Royal Swedish Academy of Sciences National Committe on Biology (1997-).

Major funding (Principal Investigator): Swedish NFR (SEK 900,000/yr for 1997 to 1999).
Publications: Over 300 scientific papers, reviews and reports in the area of developmental neuroscience

SELECTED LIST OF PAPERS PUBLISHED BY TED EBENDAL

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Ericsson, A. 1988. Structure and expression of ß-nerve growth factor in the rat central nervous system. In: Neural Development and Regeneration (ed. A. Gorio et al.). NATO ASI Series, Vol. H 22: 245-256. Springer, Berlin.

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Molecular cloning and neurotrophic activities of a protein with structural similarities to nerve growth factor: Developmental and topographical expression in the brain

(nerve growth factor family/cDNA/neurotrophic factor/hippocampal neurons/nerve growth factor receptor binding)

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ABSTRACT **We have used a pool of degenerate oligonucleotides representing all possible codons in regions of homology between brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) to prime rat hippocampal cDNAs in the polymerase chain reaction. The amplified DNA included a product with significant similarity to NGF and BDNF, which was used to isolate a 1020-nucleotide-long cDNA from a rat hippocampal library. From the nucleotide sequence, a 282-amino-acid-long protein with ≈45% amino acid similarity to both pig BDNF and rat NGF was deduced. In the adult brain, the mRNA for this protein was predominantly expressed in hippocampus, where it was confined to a subset of pyramidal and granular neurons. The developmental expression in brain showed a clear peak shortly after birth, 1 and 2 weeks earlier than maximal expression of BDNF and NGF, respectively. It was also expressed in several peripheral tissues with the highest level in kidney. The protein, transiently expressed in COS cells, was tested on chicken embryonic neurons and readily stimulated fiber outgrowth from explanted Remak's ganglion and, to a lesser extent, the nodose ganglion. A weak, but consistent, fiber outgrowth response was also seen in the ciliary ganglion and in paravertebral sympathetic ganglia. Moreover, the protein displaced binding of NGF to its receptor, suggesting that it can interact with the NGF receptor. Thus, this factor, although structurally and functionally related to NGF and BDNF, has unique biological activities and represents a member of a family of neurotrophic factors that may cooperate to support the development and maintenance of the vertebrate nervous system.**

During development of the vertebrate nervous system, a vast overproduction of neurons is compensated for by naturally occurring neuronal death, which is regulated by their targets (1). Within the targets, specific proteins, referred to as neurotrophic factors, are produced in limiting amounts and the release of these proteins is believed to regulate both the timing and the extent of innervation (2).

In the peripheral nervous system, the most well-characterized neurotrophic factor, nerve growth factor (NGF), supports the development of sympathetic and neural crest-derived sensory neurons, and in the adult the maintenance of the sympathetic nervous system is critically dependent on NGF (3, 4). In agreement with a trophic role of NGF for adult sympathetic neurons, the levels of both NGF mRNA and protein correlate with the density of sympathetic innervation (5, 6). NGF mRNA and protein have also been found in the brain, with the highest levels in hippocampus and cerebral cortex, to which the major cholinergic pathways in the brain project (7–10). Basal forebrain cholinergic neurons can be prevented from dying after axonal transection by addition of NGF (11–15) and they respond to NGF *in vivo* by a marked increase in fiber outgrowth (16).

In addition to NGF, one other protein, termed brain-derived neurotrophic factor (BDNF), has been shown to be present in low amounts (17), secreted from cells (18), and to support survival of embryonic sensory neurons *in vivo* (19). In common with NGF, BDNF supports the survival of neural crest-derived embryonic sensory neurons *in vitro*, but nonoverlapping trophic activities are suggested by the finding that BDNF also supports placode-derived neurons from the nodose ganglia and retinal ganglion cells (20, 21), which are less sensitive to NGF (22, 23). Regulation of neuronal survival *in vivo* in the brain by BDNF has not yet been demonstrated, although its sites of synthesis have recently been mapped by *in situ* hybridization where a high level of labeling was found in hippocampal neurons (24).

NGF is synthesized as a preproprotein and the structure of both the precursor and the mature protein has been deduced from cDNA and genomic clones (25, 26). More recently, a genomic clone has been isolated for porcine BDNF (18). Of considerable interest is the finding that the mature BDNF and NGF proteins show striking amino acid similarities, suggesting that they are structurally related and may be members of a family of neurotrophic factors (18).

In this study, we report on the cloning and expression of an additional member of the NGF family.¶ Due to its restricted expression in the brain, being mostly confined to a subset of pyramidal and granular neurons in the hippocampus, we have named this protein hippocampus-derived neurotrophic factor (HDNF).

MATERIALS AND METHODS

RNA Preparation, Molecular Cloning, and DNA Sequencing. Polyadenylylated RNA [poly(A)$^+$] was prepared as described (27). For cloning, rat hippocampus poly(A)$^+$ RNA (5 μg) was used as a template for synthesis of single-stranded cDNA using Moloney murine leukemia virus reverse transcriptase (Pharmacia). Six separate mixtures of 28-mer oligonucleotides representing all possible codons corresponding to the amino acid sequence KQYFYET (5′-oligonucleotide) and WRFIRID (3′-oligonucleotide) were synthesized on an Applied Biosystems A381 DNA synthesizer. The 5′-oligonucleotide contained a synthetic *Eco*RI site and the 3′-oligonucleotide contained a synthetic *Hin*dIII site. Each mixture of oligonucleotides was then used to prime the amplification of hippocampal cDNA (25 ng) by the polymer-

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Abbreviations: NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; HDNF, hippocampus-derived neurotrophic factor; PCR, polymerase chain reaction.

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¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. M34643).

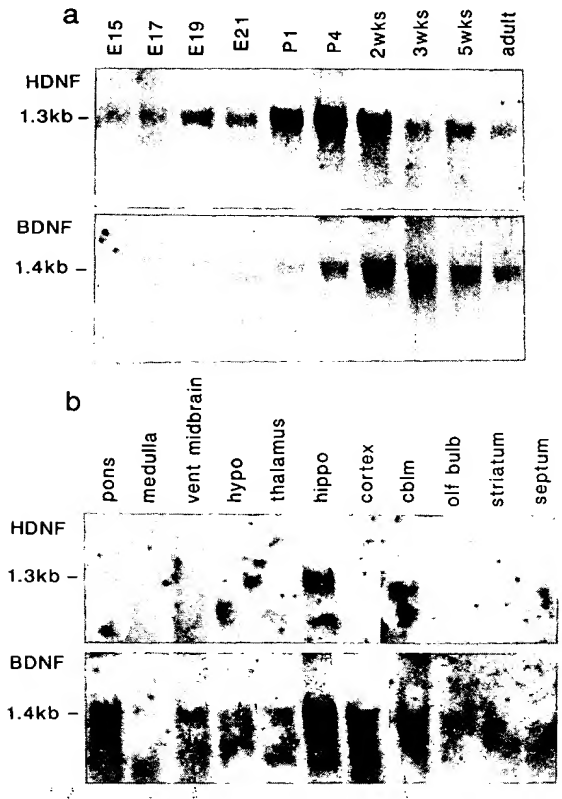

FIG. 2. Developmental and regional expression of HDNF and BDNF mRNA in rat brain. (*a*) Poly(A)$^+$ RNA (20 μg per slot) isolated from Sprague–Dawley rat brain at the indicated developmental stages was hybridized to the indicated probes (HDNF and BDNF). Adult rats were 12 weeks old. E, embryonic day; P, postnatal day; wks, weeks. (*b*) Same analysis as in *a* using poly(A)$^+$ RNA (20 μg per slot) isolated from the indicated regions of adult male Sprague–Dawley rat brain. Medulla, medulla oblongata; hypo, hypothalamus; hippo, hippocampus; cortex, cerebral cortex; cblm, cerebellum; olf, olfactory bulb.

brain showed remarkable regional specificity with high levels in hippocampus compared with other brain regions analyzed (Fig. 2*b*). In fact, cerebellum was the only other region where HDNF mRNA was clearly detected, with the exception of cerebral cortex, which showed a weak signal. BDNF mRNA was more widely distributed in rat brain, although hippocampus also contained the highest amount, followed by cerebral cortex, pons, and cerebellum (Fig. 2*b*).

Neurons Expressing HDNF and BDNF mRNA Are Located in a Distinct Topographical Arrangement in Hippocampus. Anterior sections of the dorsal hippocampus showed neurons expressing high levels of HDNF mRNA primarily confined to the medial part of CA1 and CA2 (Fig. 3 *a* and *c*). Few HDNF mRNA-expressing neurons were also found in lateral parts of CA1. Granular cells of the dentate gyrus were also highly labeled (Fig. 3*a*). CA3 and hilar cells of the dentate gyrus showed no labeling for HDNF mRNA at any level (Fig. 3*d*). No labeling was seen over any sections after hybridization to a control probe, complementary to the specific HDNF probe. Adjacent sections hybridized to a BDNF-specific probe revealed labeling over granular neurons in the dentate gyrus (Fig. 3*b*), although possibly with lower intensity than that seen after hybridization for HDNF mRNA. Strong labeling with the BDNF-specific probe was found over neurons in the hilar region (Fig. 3*e*), CA3, and part of CA2 (Fig. 3*b*). Few BDNF mRNA-expressing neurons, which appeared to be less intensively labeled, were also detected in CA1 and CA2 (Fig. 3*b*). Intensely labeled neurons were seen in claustrum, located lateral to the external capsule. This region showed no labeling for HDNF mRNA.

Neurotrophic Activities of HDNF in Explanted Chicken Embryonic Ganglia. The 1020-bp HDNF cDNA insert was cloned in the expression vector pXM (34), designed for transient expression in COS cells. Two plasmid constructs were isolated, containing the HDNF insert either in the correct or opposite orientation for translation of the HDNF protein. The latter construct was used as a negative control. Included was also a construct containing the rat NGF gene (36). The different constructs were transfected into COS cells and 3 days later conditioned medium was tested for biological activity in bioassays that measured fiber outgrowth from various chicken embryo ganglia. A marked stimulation of neurite outgrowth, consistently resulting in circular or oval fiber halos, was seen in the ganglion of Remak, a ganglionated nerve trunk in the mesorectum of the chicken embryo (38, 39) (Fig. 4*a*). Although NGF is known to stimulate the explanted ganglion of Remak (39), it was far less efficient than HDNF (Fig. 4*b*). A modest stimulation of fiber outgrowth was also seen with HDNF in the nodose ganglion, consisting of neurons exclusively derived from an epidermal placode (22)

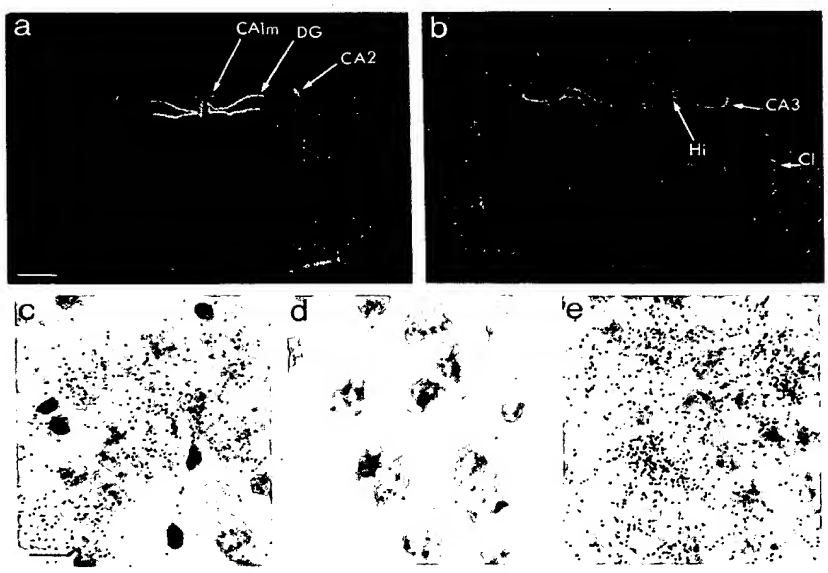

FIG. 3. Expression of HDNF and BDNF mRNA in hippocampal neurons. Rat (Sprague–Dawley) brain sections hybridized to either HDNF- or BDNF-specific oligonucleotide probes. (*a*) Autoradiogram from a section at the level of hippocampus hybridized to the HDNF-specific probe. Note labeling over medial CA1, CA2, and the dentate gyrus. (*b*) Adjacent section hybridized to a BDNF-specific probe. Note labeling over CA2 and CA3 as well as hilar cells and dentate granule layer. (*c*) Pyramidal neurons in medial CA1 labeled with the HDNF-specific probe. (*d*) Nonlabeled hilar neurons after hybridization to the HDNF-specific probe. (*e*) Hilar neurons labeled with the BDNF-specific probe. DG, dentate gyrus; CA1m, CA1 medial; Hi, hilus of dentate gyrus; Cl, claustrum. (*a* and *b*, bar = 1.3 mm; *c*–*e*, bar = 10 μm.)

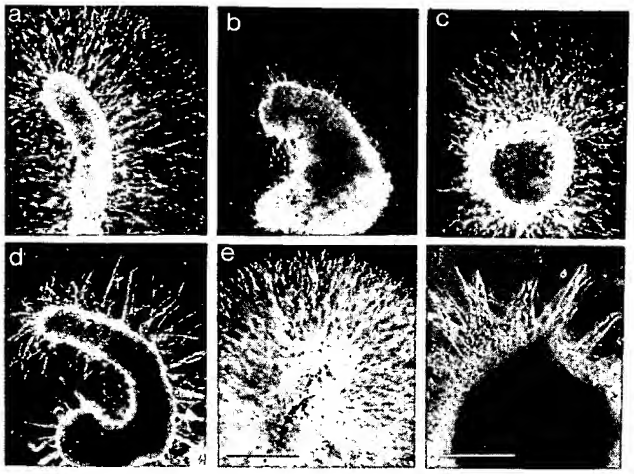

FIG. 4. Stimulation of fiber outgrowth from chicken embryonic ganglia. Biological activity of recombinant HDNF shown as effects on different nerve tissues from the chicken embryo. Remak ganglion stimulated by HDNF (*a*) or NGF (*b*). (*c*) Nodose ganglion with HDNF. Paravertebral sympathetic ganglion in response to HDNF (*d*) and recombinant rat NGF (*e*). (*f*) Ciliary ganglion with HDNF. All figures show ganglia after 1.5 days in culture. Dark-field microscopy. (Bars = 0.3 mm.)

(Fig. 4*c*). Again, HDNF was superior to NGF in evoking this response. A weak, but consistent, fiber outgrowth response with HDNF was seen in paravertebral sympathetic trunk ganglia (Fig. 4*d*), which, however, was much less pronounced compared with the massive response to rat NGF (Fig. 4*e*). In the ciliary ganglion, a weak but consistent fiber outgrowth response, manifested by the projection of short neurite fascicles, was seen with HDNF but never with NGF (Fig. 4*c*). In the dorsal root ganglia, HDNF stimulated neurite outgrowth to the same extent as NGF.

Displacement of NGF Binding to PC12 Cells by HDNF. Concentrated conditioned medium from transfected COS cells was tested for its ability to compete for binding ^{125}I-labeled NGF (^{125}I-NGF) to its receptor on PC12 cells. The concentration of ^{125}I-NGF used allowed ≈80% of the labeled NGF to be bound to the low-affinity receptor site in the absence of competition (40). Twenty-five times concentrated medium containing the HDNF protein displaced ≈70% of the labeled NGF and a 20% displacement was seen after a 25-fold dilution (Fig. 5). In contrast, 25 times concentrated medium from COS cells transfected with the HDNF cDNA in the opposite orientation did not show any displacement. Concentrated medium from cells transfected in parallel with a rat NGF gene displaced 50% of the labeled NGF when diluted 250 times.

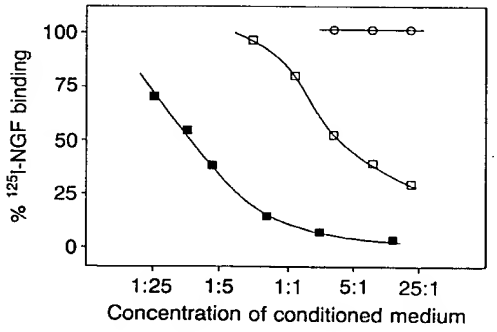

FIG. 5. Displacement of ^{125}I-NGF from its receptor on PC12 cells by HDNF and NGF. Serial dilutions of transfected COS cell medium with (□) or without (○) HDNF or containing rat NGF (■) were assayed for their ability to displace ^{125}I-NGF from its receptor on PC12 cells. Data are from two independent experiments that showed a variation of ±20%.

DISCUSSION

The cDNA clone isolated in this study encodes a protein, HDNF, with a remarkable sequence similarity to both NGF and BDNF and therefore represents an additional member of a family of neurotrophic proteins. Recently (at the time of submission of this manuscript), two groups (41, 42) independent of us isolated genomic clones for a protein (neurotrophin 3) from mouse and rat, respectively, which is identical to the neurotrophic protein characterized in this study. Our cDNA clone predicts a 282-amino-acid-long protein, which is 24 amino acids longer than the protein deduced from the genomic clones (41, 42). Two alternative start sites for translation of the NGF protein have been proposed; the first is located in a separate 5′ exon (43). The second start site, located in the 3′ exon, is also efficiently used for translation of the NGF protein (36, 44) and generates a 68-amino acid shorter protein. Thus, the structure of our cDNA clone indicates that the HDNF protein utilizes two alternative start sites for translation, located in separate exons, and suggests that the genomic organization of HDNF and NGF is very similar.

In peripheral ganglia bioassays, HDNF showed neurotrophic activities that were to some extent reminiscent of both NGF and BDNF. Thus, in similarity to BDNF (20), HDNF stimulated fiber outgrowth from the nodose ganglia and, as for NGF, evoked a fiber outgrowth response in sympathetic ganglia. In the latter case, however, the response was clearly weaker than with NGF. The partially overlapping activities seen *in vitro* may reflect a cooperation of these factors *in vivo*, where two or more proteins from the same family may support the development and/or maintenance of specific neurons. The most striking stimulation of fiber outgrowth evoked by HDNF was seen in the peripheral, autonomic, ganglion of Remak containing mostly cholinergic but also some adrenergic neurons (38, 39). This effect was clearly more pronounced than effects seen with NGF (39), suggesting that HDNF also evokes trophic responses different from both NGF and BDNF. In agreement with this, HDNF showed a weak, but consistent, neurite outgrowth response in the ciliary ganglion, which does not respond to NGF or BDNF. The ciliary ganglion is known to respond to ciliary neurotrophic factor (45), which lacks a signal sequence, but could be released by an as yet unknown mechanism (46). Thus, HDNF is the only secreted neurotrophic factor today that is known to affect fiber outgrowth, at least *in vitro*, from the ciliary ganglion.

The HDNF protein displaced ^{125}I-NGF from PC12 cells, indicating that it can interact with the NGF receptor. With the assumption that NGF and HDNF were produced in equal amounts in parallel transfections and that the conditioned medium lacks interfering substances, the interaction of NGF to its receptor was 30-fold more efficient. PC12 cells have both low- and high-affinity receptors but only the high-affinity receptor mediates a biological response (47). The fact that recombinant rat NGF readily stimulated neurite outgrowth from PC12 cells, whereas HDNF, even at 30-fold higher concentrations than NGF, did not suggests that HDNF can only interact with the NGF receptor in its low-affinity form. It therefore appears likely that the biological responses elicited by HDNF are mediated by either a separate second messenger system compared with NGF or that the HDNF receptor is different from the NGF receptor.

In similarity with NGF, HDNF mRNA was found in several peripheral rat tissues, with the highest level in kidney. Hybridization of the same filters to a rat NGF probe revealed that the level of HDNF mRNA in kidney was only slightly

higher than the levels of NGF mRNA in peripheral sympathetic target tissues, indicating that HDNF is produced in relatively small amounts in peripheral rat tissues. This is also true for the brain, and the fact that seven positive cDNA clones were isolated from 1.2×10^6 independent clones suggests that in hippocampus, containing the highest level of HDNF mRNA, this transcript constitutes ≈ 1 in every 170,000, which clearly represents a rare transcript. Thus, as in the case of NGF, HDNF may be present in limiting amounts and functions *in vivo* as a target-derived factor for a specific subset of both peripheral and central neurons. The regional distribution of HDNF mRNA in the periphery is, however, different from NGF, and, in agreement with the *in vitro* biological assays, HDNF may support a different set of peripheral neurons. Of interest is also that HDNF mRNA was found in the ovary, whereas no mRNA was detected in the testis, where both NGF and its receptor is expressed (48) and where NGF has been suggested to mediate an interaction between Sertoli cells and germ cells (49). This shows that different members of the NGF family are expressed in different reproductive tissues and suggests that they may have nonoverlapping functions outside the nervous system.

Interestingly, the three neurotrophic proteins were maximally expressed at different times of brain development with a peak of HDNF mRNA shortly after birth, BDNF mRNA around 2 weeks, and NGF mRNA around 3 weeks after birth (see ref. 8 for NGF). Moreover, the mRNA's for all three proteins were expressed in hippocampus at levels higher than in other regions, particularly in the case of HDNF. Within hippocampus, all three mRNAs were also confined to neurons (see ref. 10 for NGF) and a clear topographical division was seen, where HDNF mRNA was concentrated to pyramidal neurons in medial CA1, CA2, and granular neurons in dentate gyrus. Strongly labeled BDNF neurons were primarily seen in CA3 and the hilar region of dentate gyrus. Neurons with apparent lower levels of BDNF mRNA were seen in the dentate gyrus. The hilar region, containing neurons with high levels of BDNF mRNA, showed no labeling for HDNF mRNA.

This remarkable concentration of trophic factors in the adult hippocampus suggests that maintenance of plasticity is crucial to its function and may relate to the presumed morphological sequelae of long-term potentiation and memory consolidation processes. The intriguing temporal and spatial expression of the three neurotrophic proteins in the brain suggests that they predominantly support neuronal innervation at different times of development and that they may also exert specific trophic support for different central nervous system neurons, a possibility that will be an interesting topic for future studies.

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